IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	SCHLOTHAUER, et al	)	Examiner: Prats, F.C.
Appl. No.	:	09/720,041	)	Group Art Unit 1651
Filed	:	April 2, 2001	)	
For	:	BIOACTIVE WHEY PROTEIN HYDROLYSATE	)	

DECLARATION OF SOPHIA STATHOPOULOS

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, SOPHIA STATHOPOULOS, depose and say as follows:

1. I am a New Zealand citizen and live in Palmerston North, New Zealand.

2. I am a technical officer at Fonterra Palmerston North, New Zealand. I work from time to time under the direction of Julian Robert Reid, a Senior Research Scientist at Fonterra, Palmerston North, New Zealand. My Curriculum Vitae is attached as Exhibit "SS1".

3. I conducted the following experiment with the aim of preparing a whey protein hydrolysate according to methods described in USSN 09/720,041 followed by an enzyme inactivation step of 80°C for 20min.

4. The experiment was based on example 4 in USSN 09/720,041. The details are:

Materials

Enzyme Neutrase sourced from *Basillus subtilis*, was purchased from Novo Nordisk, Denmark. "A421 Whey Protein Concentrate" was prepared by Fonterra, New Zealand (58%protein, 30%lactose).

SS

Experimental method

A421 Whey Protein Concentrate was reconstituted with water in a stirred stainless steel beaker at 15% w/w total solids (300.06g A421 made up to 2.006.67g with Milli Q water), and placed in a 52°C waterbath. At 50°C the solution was adjusted to pH 7.02. Neutrase enzyme was prepared by adding 5mL of water to 1.044g of Neutrase. The enzyme solution was allowed to stand for 10mins. Reaction was initiated by adding the prepared enzyme to the reconstituted A421 solution (final enzyme:protein ratio of 0.6% w/w). A421 was hydrolysed for 2 h at 50°C with gentle agitation. Once the reaction was complete, equal sized portions of the A421 hydrolysate were subjected to different heat inactivation steps as follows:

1. 500mL of the hydrolysate, was heat inactivated in an 80°C waterbath (covered) for 20min. Treatment was timed from as soon as the solution reached 80°C (5min). Once treatment was complete the sample was cooled to room temperature in an ice bath.
2. 500mL of the hydrolysate, was heat inactivated at 88°C for 10 sec, using a steel coil immersed in a 95°C waterbath. The solution exiting the coil was collected in a beaker surrounded by ice.
3. 500mL of the hydrolysate, was heat inactivated at 88°C for 3 sec, using a steel coil immersed in a 95°C waterbath. The solution exiting the coil was collected in a beaker surrounded by ice.

The levels of denatured protein were measured once the heat-treated samples reached room temperature. This was done by centrifuging a sample of each hydrolysate (3000 x g for 10min) and measuring the total solids in the supernatant solution and in the precipitate fraction collected following centrifugation . The total solids present in the hydrolysate prior to heat treatments were also determined. Photographs were also taken to show visible differences between the hydrolysates inactivated using the different heat treatments.

Results

Photographs of hydrolyaste samples after heat inactivation (before and after centrifugation) are presented below.

GD.

4. I conducted a further experiment as follows:

Experimental method

A421 Whey Protein Concentrate was reconstituted with water in a stirred stainless steel beaker at 15% w/w total solids (150.4 g A421 made up to 1002.7 g with Milli Q water), and placed in a 52°C waterbath. At 50°C the solution was adjusted to pH 6.99. Neutrase enzyme was prepared by adding 5mL of water to 0.572 g of Neutrase. The enzyme solution was allowed to stand for 10mins. Reaction was initiated by adding the prepared enzyme to the reconstituted A421 solution (final enzyme:protein ratio of 0.66% w/w, the extra 0.06% enzyme was added to make up for the loss of enzyme activity over a one year storage). A421 was hydrolysed for 24 hours (according to the method of Abubakar *et al.*, 1996) at 50°C with gentle agitation. Once the reaction was complete, the A421 hydrolysate was tested for degree of hydrolysis (method in Appendix 1) and subjected to heat inactivation with the aim of heating at 96°C for 10 min (according to the method of Abubakar *et al.*, 1996). 500 mL of A421 hydrolysate was placed in a 97°C waterbath (covered). After 5 min, the hydrolysate temperature reached 88°C, but it had already formed a thick coagulant. The temperature of the coagulated hydrolysate stayed at approx. 88°C for the remaining 5 min as the coagulant formed an insulating layer around the outside of the reaction vessel, thus preventing further heat transfer during this time. Once the heat treatment was complete, the sample was rapidly cooled to room temperature in an ice bath.

The levels of denatured protein were measured once the heat-treated sample reached room temperature. This was done by centrifuging a 50 mL sample of heat-treated hydrolysate at 3000 x g for 10min. Photographs were also taken to show visible levels of denatured protein.

Results

Degree of Hydrolysis

SB

Heat Inactivation

The A421 hydrolysate formed a thick coagulant after treatment at 80°C/20 minutes (Figure 1; left hand beaker). The A421 hydrolysate treated at 88°C for 10 seconds remained soluble (Figure 1; right hand beaker). No distinct colour change was observed.

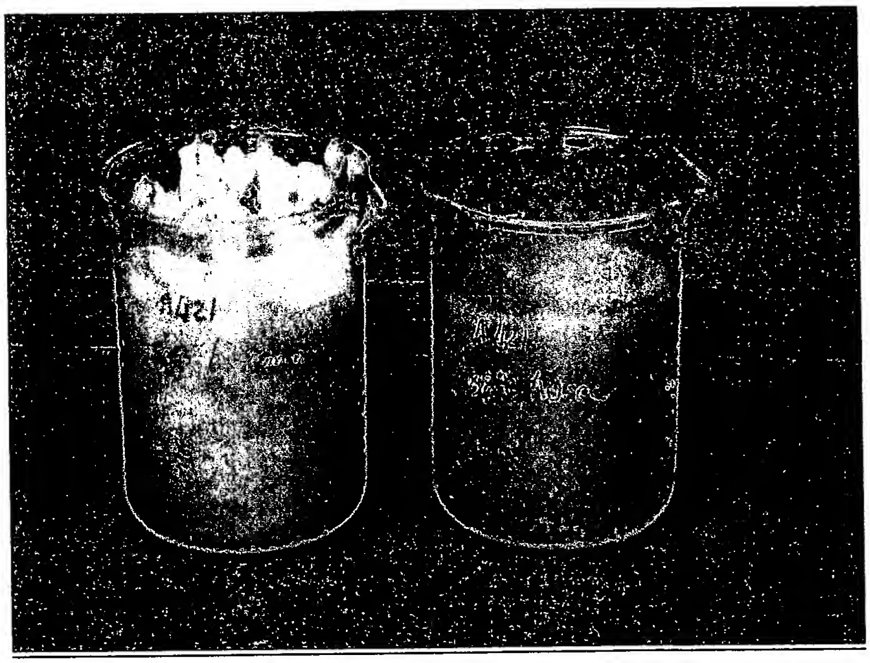

Figure 1: Heat inactivation of A421 WPC hydrolysate using two different methods; 80°C/20min (left) versus 88°C/10sec (right).

Centrifugation

Samples (50 mL) of each of the three inactivated hydrolysates were centrifuged. A distinct thick pasty white precipitate of approximately 30 mL was observed after centrifugation of the hydrolysate inactivated at 80°C for 20 minutes (Figure 2, right hand pair of tubes). However, only 3 mL of precipitate was observed with the hydrolysate inactivated at 88°C / 3 sec (Figure 2).

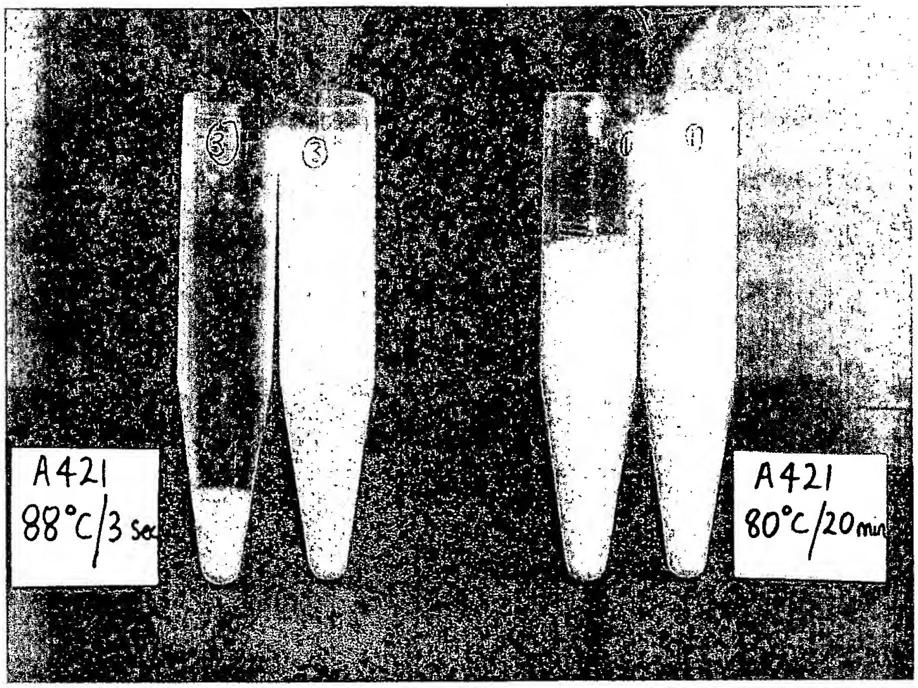

Figure 2: Heat inactivation of A421 WPC hydrolysate using two different methods. Samples are shown of A421 hydrolysate inactivated at 80°C/20min (right) and at 88°C/3sec (left). For each pair of tubes, one is shown with the supernatant removed.

Percentage Denaturation

Using the total solids method, the percentage denaturation of the sample inactivated at 80°C/20 minutes was 66.5%, while for the sample inactivated by heating at 88°C/10 seconds the percentage denaturation was 1.8%

Conclusions

The use of a heat step of 80°C/20 minutes to inactivate the enzyme in a WPC hydrolysate prepared according to the method in example 4 of USSN 09/720,041 results in significant and unacceptable levels of denaturation (66.5%). This level of denaturation results in a coagulated product (Figure 1) that is impossible to process further and is therefore of no commercial value. On the other hand, inactivation conditions of 88°C/10 seconds, results in extremely low levels of denaturation (1.8%), and the product can easily be spray dried to produce a soluble, commercially valuable powder.

the coagulant. Thus, if it had been possible to apply the full heat treatment (*i.e.* 96°C), the level of denaturation would have been even higher than the 51% that we observed. Using the hydrolysis method given in example 4 of USSN 09/720,041, but with an extended 24 hour hydrolysis step (as decribed by Abubakar *et al.*, 1996) a relatively low degree of hydrolysis (6.3%) was reached. Therefore, some undigested protein along with a significant amount of semi-digested protein (large protein fragments) remained after the 24 hour hydrolysis step. Under the harsh inactivation conditions of Abubakar *et al.* (1996) these components formed the large precipiate shown in Fig. 4. This level of denaturation results in a coagulated product (Figure 3) that is impossible to process further and is therefore of no commercial value. Again, this contasts with the results obtained using inactivation conditions of 88°C/10 seconds, where extremely low levels of denaturation (1.8%) occur (see point 4 above).

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that willful, false statements may jeopardize the validity/enforceability of the application or any patent issued thereon

Dated: 18/03/04 Signature: [signature]

Sophia Stathopoulos

SS.

The degree of hydrolysis after 24 hours was determined to be 6.3% (see Appendix for method).

Heat Inactivation

The 24 hour A421 hydrolysate formed a thick coagulant after treatment at 88°C/10 minutes (Figure 3). No distinct colour change was observed.

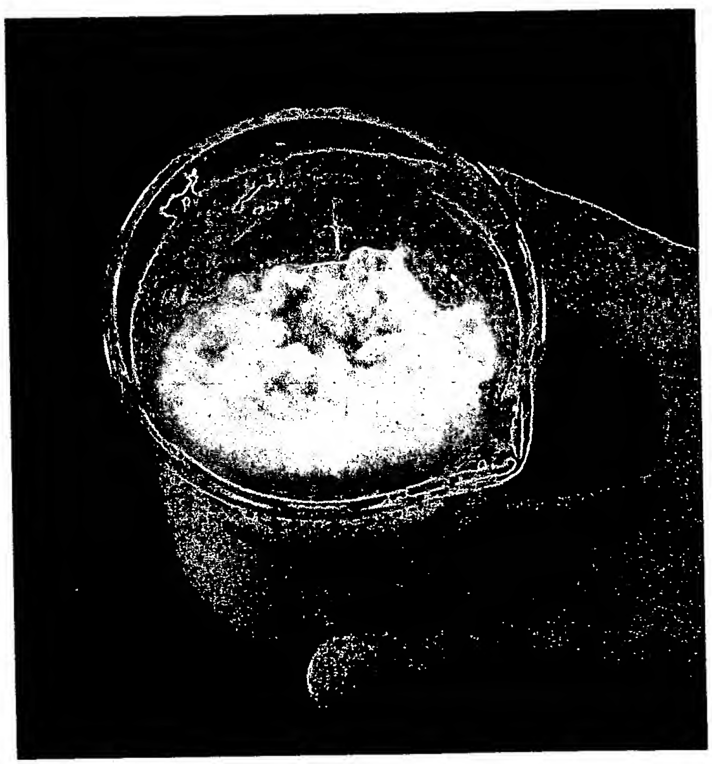

Figure 3: Heat inactivation at 88°C/10min of A421 WPC 24 h hydrolysate.

Centrifugation

A distinct thick pasty white precipitate of approximately 23 mL was observed after centrifugation of 50 mL of the hydrolysate inactivated at 88°C for 10 minutes (Figure 4).

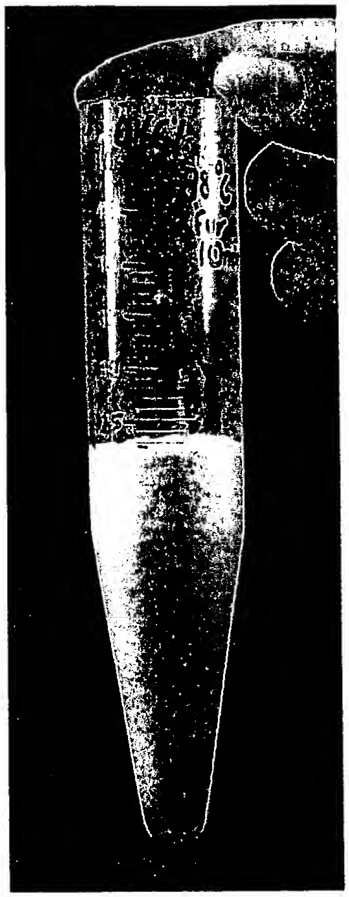

Figure 4: Heat inactivation of A421 WPC 24 h hydrolysate at 88 °C/10min. 50 mL of heat-inactivated hydrolysate was centrifuged - the supernanatant has been removed.

Percentage Denaturation

Using the results from centrifugation, the percentage denaturation of the sample inactivated at 88°C/10 minutes was calculated to be 51%.

Conclusions

Abubakar et al. (1996) used a 96°C/10 min heat treatment to inactivate hyrolysates. However, these authors' used very small-scale digests of just 0.01-0.1 g protein in a 10 mL reaction volume. It is very difficult to apply this method to significantly larger reaction volumes due to the severe protein denaturation that results. In a 10 min heat step, our hydrolysate only reached 88°C due to the heat-insulating nature of

THIS is the appendix 1 referred to in paragraph 4 of the annexed declaration of SOPHIA STATHOPOULOS declared at Palmerston North this 18th day of March 2004 in relation to USSN 09/720,041.

SS

Appendix 1

Degree of Hydrolysis by the Modified O-phthaldialdehyde (MOPA) Method

Reference: Frister H, Meisel H & Schlimme E (1988)
Freesenius Z Anal Chem. 330, 631-633

Principle: This method is used to determine the degree of hydrolysis (DH) of hydrolysed protein products. The sample is diluted in a MOPA buffer and the absorbence converted to μmol of amino groups by a glycine standard curve at 340nm

Apparatus: Spectrophotometer operating at 340nm.

Materials and Reagents:

Reagents

- Di-sodium tetraborate (MW 381.36)
- Sodium dodecyl sulfate (SDS) (MW 288.4)**
- 2-(Dimethylamino) ethanethiol hydrochloride (MW 141.67)**
- O-phthaldialdehyde (MW 134.1)**
- Glycine (MW 75.1)
- Methanol (Analar)
- Milli-Q water

MOPA buffer

	%
250mL of 0.1M (38.136 g/l) di-sodium tetraborate	50.0
50 mL of 10% sodium dodecyl sulfate (SDS)	5.0
1g of 2-(Dimethylamino) ethanethiol hydrochloride	0.2

58

Mix above ingredients and bring to 500mL total volume with MilliQ water.
Store buffer in refrigerator. The buffer has limited shelf-life and should be used within a period of ten days.

Redissolve the SDS by gentle warming prior to use.

O-phthaldialdehyde (OPA) reagent

Dissolve 40 mg of OPA reagent in 1 mL of methanol, then make up to 50 mL total volume with MOPA buffer. 3 mL of OPA reagent is required for each sample and each standard assayed.
This reagent should be made fresh daily. Make just before use as it is affected by light.

Standard curve

1. Dissolve 3.0028 g/l of glycine in MilliQ water to make a 0.04 M solution and store in a refrigerator.
2. Dilute 1:10 in MOPA buffer to make 0.004 M solution (0.2 mL of 0.04 M glycine solution and 1.8 mL of MOPA buffer gives sufficient for the standard curve dilutions).
3. Prepare the following dilution series:

0.004 M glycine (mL)	**MOPA buffer (mL)**	**µmol/200µL glycine**
0.0625	0.9375	0.05
0.125	0.875	0.10
0.1875	0.8125	0.15
0.25	0.75	0.20
0.375	0.625	0.30

Sample preparation

Dilute the samples in MOPA buffer to give a final concentration between 0.3 - 0.5mg/mL approx and mix well. Use 1mg/mL for lightly hydrolysed samples.
For powdered samples, weigh 3-5mg and add 10 mL of MOPA buffer. Record the exact weight of powder in g. For liquid samples record the dilution factor.

Procedure

1. Blank the spectrophotometer on OPA reagent.
2. Add 200µL of sample to cuvette and then add 1.5 mL of OPA reagent.

SB.

3. Read the absorbance at 340 nm after exactly 2 minutes.
4. Test the standards and samples in duplicate.

Calculations

1. Average all duplicate absorbances of samples and standards.
2. Plot µmol amino groups (glycine) versus absorbance for the standard curve.
3. Check the linearity of the standard curve and perform linear regression on the points in the linear range. Obtain the regression equation of the curve in the form:

$$\text{Absorbance} = (A * \mu\text{mol amino groups}) + B$$

Where: A is the slope of the curve
B is the constant
* multiplication

Note: the curve is usually linear throughout the dilution series.

4. Convert the absorbances of the samples to µmol amino groups (C). Check that the absorbances are in the linear range

$$C = (\text{Absorbance}_{\text{sample}} - B)/A$$

5. Calculate the weight of protein per sample in g in the OPA reaction mixture (D)

$$D = (\text{wt sample}/10)*0.2*\%\text{protein in sample}/100$$

6. Calculate the µmol amino groups /g protein (H) for each sample

$$H = C/D$$

7. Calculate the degree of hydrolysis (DH) by the following formula. The formula subtracts the theoretical contribution of lysine side-chains (E) and expresses the remaining amino groups as a percentage of the total theoretical number of peptide bonds/g protein (H_{total})

$$DH = (H - E)/ H_{total}*100$$

E and H_{total} are defined for each substrate in the table below.

S.D.

Protein substrate	**H_{total} (µmol NH_2/g protein)**	**E (µmol NH_2/g protein)**
Casein	8200	560
Acid wpc	8800	650
Cheese/rennet wpc	8800	570
Lactalbumin	8800	730
TMP	8320	578

THIS is the exhibit marked "SS" referred to in paragraph 2 of the annexed declaration of SOPHIA STATHOPOULOS declared at Palmerston North this 18th day of March 2004 in relation to USSN 09/720,041.

SS.

Curriculum Vitae

NAME:	**SOPHIA STATHOPOULOS**
CURRENT EMPLOYER:	FONTERRA RESEARCH CENTRE LIMITED Private Bag 11 029 Palmerston North NEW ZEALAND Technical Officer.
DEGREES:	COMPLETING MASTERS IN BIOTECHNOLOGY (final year) at Massey University, Palmerston North, New Zealand. BACHELOR OF SCIENCE Biochemistry, Physiology, Genetics and Molecular Biology (1996) at Victoria University, Wellington, New Zealand.

WORK EXPERIENCE

Over the past two years I have been working towards a Masters in Biotechnology foccusing on the use of membrane bioreactors for the dairy industry. Currently I am working with protein purification and protein hydrolysates.

I have worked for FRC as a technician for approx. 6 years, assisting in research associated with:

1. Cheese, milk powders, and whey products
2. polysaccharide and yeast applications, as well as analytical method development on certain cheese products
3. Protein hydrolystaes

COURSES

- Masters programme (2002-2004)
- Worked for 3 weeks at University of Calabria (Italy) to gain further understanding of membrane bioreactors
- Particle Size Measurements Course, Massey University, Palmerston North, New Zealand; 2001
- Protein Purification Course, University of Waikato, Hamilton, New Zealand; 2000

SS